

# In Vitro Dissolution of Uranium Oxide by Baboon Alveolar Macrophages

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*In vitro* cellular dissolution tests for insoluble forms of uranium oxide are technically difficult with conventional methodology using adherent alveolar macrophages. The limited number of cells per flask and the slow dissolution rate in a large volume of nutritive medium are obvious restricting factors. Macrophages in suspension cannot be substituted because they represent different and poorly reproducible functional subtypes with regard to activation and enzyme secretion. Preliminary results on the dissolution of uranium oxide using immobilized alveolar macrophages are promising because large numbers of highly functional macrophages can be cultured in a limited volume. Cells were obtained by bronchoalveolar lavages performed on baboons (*Papio papio*) and then immobilized after the phagocytosis of uranium octoxide ( $U_3O_8$ ) particles in alginate beads linked with  $Ca^{2+}$ . The dissolution rate expressed as percentage of initial uranium content in cells was  $0.039 \pm 0.016\%$ /day for particles with a count median geometric diameter of  $3.84 \mu m (\sigma_g = 1.84)$ . A 2-fold increase in the dissolution rate was observed when the same number of particles was immobilized without macrophages. These results, obtained *in vitro*, suggest that the  $U_3O_8$  preparation investigated should be assigned to inhalation class Y as recommended by the International Commission on Radiological Protection. Future experiments are intended to clarify this preliminary work and to examine the dissolution characteristics of other particles such as uranium dioxide. It is recommended that the dissolution rate should be measured over an interval of 3 weeks, which is compatible with the survival time of immobilized cells in culture and may reveal transformation states occurring with aging of the particles.

## Introduction

Industrial particles produced in the manufacture of nuclear fuel compounds may be inhaled accidentally by workers. A good knowledge of the toxicokinetic and metabolic behavior of these radioactive compounds is required to interpret routine bioassay measurements, especially the rate of urinary and fecal excretion. These analyses can reveal the clearance of the compound from the body and the long-term distribution among tissues, which are important data in evaluating the cumulative radiation dose from internal exposure.

When uranium compounds are inhaled, both chemical- and radiation-induced tissue damage may occur (1,2). Although the chemical toxicity to the kidney is predominant with soluble forms of uranium compounds, the dissolution rate is an important characteristic of less soluble uranium particles in predicting the long-term radiotoxic effects after inhalation.

Industrial uranium oxide particles were produced by different chemical and physical processes to give a range of solubility characteristics (3,4). Using chemical acidified solvents or biological fluid simulants, few techniques are available to estimate reliably the solubility of industrial particles (5). To

evaluate the variation in long-term pulmonary clearance and urinary excretion, these techniques are not always appropriate and often provide results that are inconsistent with those obtained from animal experiments (6). The use of a cellular *in vitro* technique with alveolar macrophages seemed more likely to predict the long-term dissolution rates of particles, especially for moderately soluble and insoluble forms (7), and also seemed more appropriate for predicting clearance after inhalation. We used this *in vitro* method to determine the intracellular dissolution rate of one form of uranium octoxide.

## Methods

### Uranium Particles

Uranium octoxide ( $U_3O_8$ ) was produced in Franco-Belge de Fabrication du Combustible manufacture (FBFC; France). We segregated particles by density gradient and centrifugation in plasmagel (Bellon, France) to obtain a polydisperse particle suspension with a count median diameter of  $3.84 \mu m$  and a geometric standard deviation of 1.84. These particles readily deposit in the alveolar region of the lung.

### Alveolar Macrophages

We recovered alveolar macrophages by bronchoalveolar lavage of baboons as described previously by Nolibet et al. (8). The baboons were first anesthetized by intramuscular injection of

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ketamine (Imalgene 500, Iffa Merieux), 10 mg/kg, and diazepam (Valium, Roche), 0.5 mg/kg, before lavaging one lung with a Carlen's double-lumen catheter. Between  $1$  and  $2 \times 10^8$  cells were usually obtained by successive injection of saline solution.

## Particle Phagocytosis and Cell Culture

The  $U_3O_8$  particle suspension was mixed and incubated with alveolar macrophages (about 5 particles per cell, equivalent to less than 10 pg of uranium per cell) for 1 hr while stirring. At the end of incubation, we rinsed the cells with 199 culture medium (Gibco) and centrifuged them before resuspension in medium with 10% fetal calf serum (FCS) (Gibco). We dissolved sterilized alginate in the medium to obtain an alginate concentration of 2%. The cell suspension was added to alginate solution to give a final alginate concentration of 1.5%. Alginate network macrobeads (diameter = 2.5 mm) were formed by extrusion through a fine catheter and gelled in a medium containing 10 mM of calcium. Three hours later, we changed the medium to a culture medium containing only 8 mM calcium. Each macrobead contained approximately  $200 \times 10^3$  cells.

Culture flasks (250 mL) containing immobilized cells in alginate beads in 199 culture medium, supplemented with appropriate amounts of FCS and antibiotics (Gibco), were put in an incubator at 37°C in a 5%  $CO_2$  atmosphere. In addition, particles were immobilized alone in alginate macrobeads and used to measure dissolution rates in the absence of alveolar cells (control).

Each day we removed the culture medium and filtered it using 0.45- $\mu$ m filters (Millipore, HAWP). Fresh medium was added daily during all the remaining time in culture. Uranium analyses were made on the different samples of medium and on the different filters pooled over three filtrations.

We took 10 macrobeads at the beginning of the dissolution test and analyzed them to estimate the initial amount of nondissolved uranium. The same measurement was made for all the beads at the end of the dissolution test to evaluate the nonsolubilized fraction of uranium remaining.

To test the integrity of the cellular functions of macrophages after cell immobilization, some alginate beads were dissolved in ethylenediaminetetraacetic acid (EDTA) solution at a concentration of 10 mM, as described by Lirsac et al. (7). Two criteria were used: the percentage of adherent cells after 2 hr in culture and the chemoluminescence of macrophages released from the beads. We measured chemoluminescence with luminol (Sigma) in the presence of phorbol myristic acetate (PMA; Sigma) or Zymosan (Sigma) opsonized with baboon serum, using a LKB 1251 luminometer.

## Measurement of Uranium

We measured uranium in the different supernatants and filters by fluorimetry (9,10). Before measurement, the culture supernatants were evaporated and the filters were mineralized with 4 N nitric acid, evaporated a second time, and redissolved in 2.5 N nitric acid. The detection limit for uranium was 5  $\mu$ g/L.

## Results

### Alveolar Macrophages and Particle Phagocytosis

Alveolar macrophages were recovered from the lung lavage fluid of two baboons, one male and one female. The initial concentrations of uranium were 55  $\mu$ g and 36  $\mu$ g of uranium per  $10^6$  cells, respectively. After phagocytosis of uranium particles, 80–83% of viable cells were found.

### Cell Culture

Three similar dissolution tests were performed with the same sample of  $U_3O_8$ . For each test, the particles were immobilized alone or after phagocytosis by baboon alveolar macrophages. One culture of macrophages was contaminated by bacteria, and the results obtained were omitted in estimating the daily dissolution rate of uranium.

In one case,  $55 \times 10^6$  cells were immobilized after uranium particle phagocytosis and  $190 \times 10^6$  cells in the second test. The adherence of cells released from alginate beads (60% of cells recovered), 14 days after phagocytosis and immobilization, shows the good viability of macrophages in culture. However, chemoluminescence with PMA or Zymosan was not observed after 10 days in culture.

### *In Vitro* Dissolution of Uranium Octoxide Particles

The daily dissolution rate of uranium octoxide phagocytized by baboon alveolar macrophages during the first 10 days of culture is shown in Figure 1. During the last phase of cell culture, from 10 to 15 days, the amount of dissolved uranium in the medium was not detectable.

Figure 1 shows that the *in vitro* dissolution rate of  $U_3O_8$  particles by baboon alveolar macrophages was low. However, 49% of the total amount of uranium was dissolved during the first 2 days of the dissolution test (Fig. 2). From day 2 to day 10 after immobilization, the dissolution rate remained constant, with a mean of  $3.9 \pm 1.6 \times 10^{-2}$ %/day.

The static dissolution of the uranium octoxide particles immobilized alone in the alginate beads is faster than the intracellular dissolution after their phagocytosis by the macrophages. Figure 3 shows that the dissolution rate remained higher for the static treatment of particles by medium than intracellular dissolution, not only during the first 2 days corresponding to the rapid phase of dissolution, but also during all the 10 days of dissolution test. These results gave a dissolution half-time of 1280 days for  $U_3O_8$  particles phagocytized by alveolar macrophages and 650 days for particles immobilized alone in alginate beads.

## Discussion and Conclusion

When virtually insoluble particles are inhaled, clearance from the pulmonary region occurs predominantly by mechanical transfer to the gastrointestinal tract. Under these circumstances, the urinary excretion rate cannot be correlated with the dissolution rate of these particles (6,11). This fact hinders the use of *in vitro* dissolution tests for interpreting routine bioassay data after accidental exposure (6). However, the dissolution test can be used to estimate the more soluble fraction of a mixed uranium compound such as yellowcake (12,13) or the variable solubility

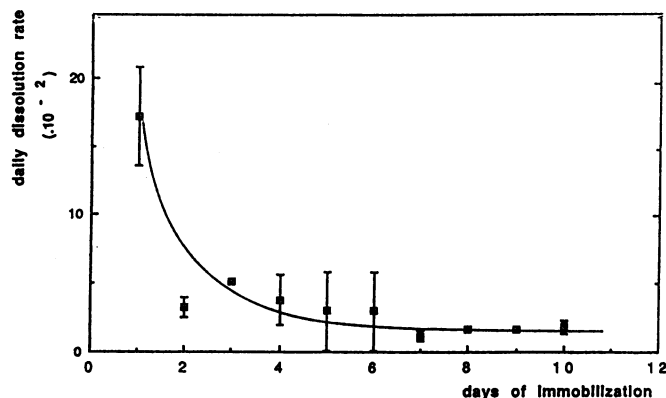


FIGURE 1. *In vitro* dissolution rate of uranium octoxide particles phagocytized by baboon alveolar macrophages (expressed as percentage of initial uranium content in alveolar macrophages per day).

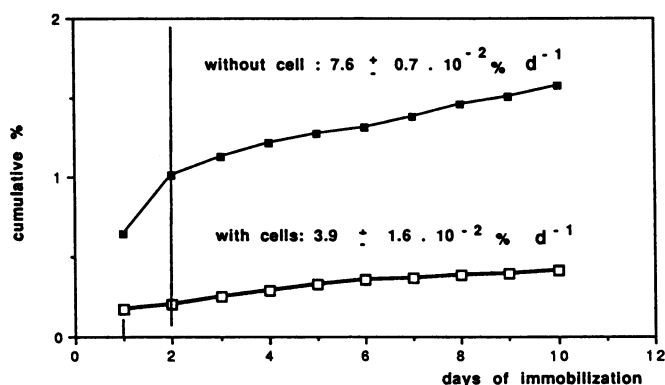


FIGURE 2. *In vitro* dissolution of uranium octoxide particles phagocytized or not phagocytized by baboon alveolar macrophages (cumulative percentage expressed as percentage of initial uranium content).

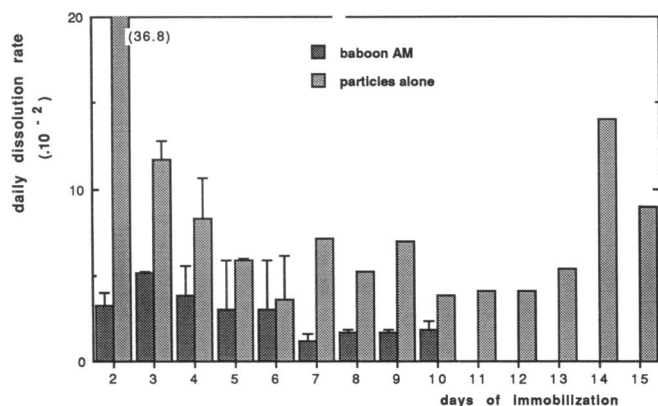


FIGURE 3. *In vitro* dissolution rate of uranium octoxide particles phagocytized by baboon alveolar macrophages (AM) or immobilized alone in alginate beads (expressed as percentage of initial uranium content per day).

rate of uranium oxides in relation to the different process lines used in the manufacture of nuclear fuel (3,4). The results of the

dissolution test might be used to modify the general assumption given by the recommendations of the ICRP (14) concerning the annual limit on intake of the uranium oxides, as suggested by Schieferdecker et al. (15), in relation to the specific workplace parameters of the compound.

The *in vivo*-like solubility tested by *in vitro* acellular tests could be influenced by different parameters that could introduce discrepancies between different workers investigating identical compounds (16,17). These variations relate to the experimental protocol, oxygenation of the solvents, etc. (18,19), or to the physical characteristics of the compounds (3). The *in vitro* technique, using specialized cells such as alveolar macrophages, markedly reduced the influence of these different parameters because it simulated the long-term *in vivo* intracellular dissolution of particles deposited in alveoli and phagocytized by macrophages (16,17,20).

In the present study, a decrease in the solubilized fraction of uranium in the culture medium was observed when particles were contained in the phagolysosomes of the macrophages. This observation could support the hypothesis of the precipitation of soluble material in the cell. For example, Galle and Berry (21) have demonstrated that the soluble fraction of uranium can be precipitated as uranium phosphate by acid phosphatase activity encountered in the lysosome of the cells. Thus, this methodology might give important information for predicting the long-term behavior of the inhaled compound in the lung.

Under normal circumstances, macrophages can only be maintained in culture for 1 or 2 weeks. The immobilization technique of cells in alginate beads permits not only an increase in cell density, but also enables cell survival for about 3 weeks. These characteristics provide a useful method to evaluate the intracellular dissolution rate of poorly soluble particles. The results show that the solubilized fraction of uranium decreased rapidly to below the detection limit after 10 days in culture when  $U_3O_8$  particles were phagocytized with a concentration of five particles per cell (about 10 pg uranium per cell). Preliminary results to investigate the cytotoxicity of uranium oxide particles indicated that a concentration of 20–25 pg per cell of uranium octoxide did not induce a dramatic cytotoxic effect on rat alveolar macrophages.

The *in vitro* technique described here can be improved further if alveolar macrophages can be maintained in culture for even longer periods and the detection limit of uranium decreased. A detection limit of about  $1 \times 10^{-3} \mu\text{g/L}$  of uranium in the culture medium should be possible with time-resolved laser-induced spectrofluorimetry (22).

In conclusion, it is difficult to compare directly the results obtained in this study with the variety of results obtained by other investigations of the dissolution rate of uranium octoxide. However, for this preparation the dissolution rate of  $3.9 \times 10^{-2} \%/ \text{day}$  corresponds to an intracellular dissolution half-time of 1280 days seems to be in agreement with other studies and with the recommendation of the ICRP (23) for uranium compounds assigned to inhalation class Y. The results of the intracellular dissolution could be used to predict the long-term lung clearance of industrial particles inhaled accidentally.

Moreover, this technique might permit the study of intracellular dissolution mechanisms, cytotoxic effects, and chemical and physical modifications of the phagocytized particles induced by lysosomal enzymes of the alveolar macrophage. This method is actually performed with uranium dioxide compounds.

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